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Abstract: Neutrophil extracellular trap (NET) formation is described as a tool of the innate host defence to fight against invading pathogens. Fibre-like DNA structures associated with proteins such as histones, cell-specific enzymes and antimicrobial peptides are released, thereby entrapping invading pathogens. It has been reported that several bacteria are able to degrade NETs by nucleases and thus evade the NET-mediated entrapment. Here we studied the ability of three different Yersinia serotypes to induce and degrade NETs. We found that the common Yersinia enterocolitica serotypes O:3, O:8 and O:9 were able to induce NETs in human blood-derived neutrophils during the first hour of co-incubation. At later time points, the NET amount was reduced, suggesting that degradation of NETs has occurred. This was confirmed by NET degradation assays with phorbol-myristate-acetate-pre-stimulated neutrophils. In addition, we found that the Yersinia supernatants were able to degrade purified plasmid DNA. The absence of Ca(2+) and Mg(2+) ions, but not that of a protease inhibitor cocktail, completely abolished NET degradation. We therefore postulate that Y. enterocolitica produces Ca(2+)/Mg(2+)-dependent NET-degrading nucleases as shown for some Gram-positive pathogens.

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Yersinia enterocolitica-mediated degradation of neutrophil extracellular traps (NETs)

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One sentence summary: This is the first experimental proof that members of the *Enterobacteriaceae* family are able to degrade NETs, possibly due to a nuclease.

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ABSTRACT

Neutrophil extracellular trap (NET) formation is described as a tool of the innate host defence to fight against invading pathogens. Fibre-like DNA structures associated with proteins such as histones, cell-specific enzymes and antimicrobial peptides are released, thereby entrapping invading pathogens. It has been reported that several bacteria are able to degrade NETs by nucleases and thus evade the NET-mediated entrapment. Here we studied the ability of three different Yersinia serotypes to induce and degrade NETs. We found that the common *Yersinia enterocolitica* serotypes O:3, O:8 and O:9 were able to induce NETs in human blood-derived neutrophils during the first hour of co-incubation. At later time points, the NET amount was reduced, suggesting that degradation of NETs has occurred. This was confirmed by NET degradation assays with phorbol-myristate-acetate-pre-stimulated neutrophils. In addition, we found that the Yersinia supernatants were able to degrade purified plasmid DNA. The absence of Ca^{2+} and Mg^{2+} ions, but not that of a protease inhibitor cocktail, completely abolished NET degradation. We therefore postulate that *Y. enterocolitica* produces Ca^{2+}/Mg^{2+}-dependent NET-degrading nucleases as shown for some Gram-positive pathogens.

Keywords: host innate immune response; NETosis; Gram-negative bacteria

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INTRODUCTION

Upon encountering invading pathogens, the innate immune response is central to controlling bacterial infections. Neutrophils are among the first cells responding to bacterial infections. In addition to their two well-known functions of phagocytosis (Metschnikow 1891) and degranulation (Lehrer and Ganz 1999), a third function of neutrophils was discovered by Brinkmann *et al.* (2004); the release of neutrophil extracellular traps (NETs), also called NETosis (Wartha and Henriques-Normark 2008). NETs allow the host innate immune system to entrap various pathogens and prevent them from spreading in the host (Brinkmann *et al.* 2004). They consist of DNA fibres associated with histones, antimicrobial peptides and granular proteins (e.g. myeloperoxidase or elastase) (von Köckritz-Blickwede and Nizet 2009). To release NETs, the neutrophils are activated by microbial pathogens or various other stimuli such as lipopolysaccharide, interferin (IFN)-α/γ + C5a or granulocyte-macrophage-colony-stimulating-factor (GM-CSF) + C5a and chemical substances such as phorbol 12-myristate 13-acetate (PMA) (Brinkmann *et al.* 2004; Martinelli *et al.* 2004; Fuchs *et al.* 2007). Upon activation, the nuclear and granular components all together are released into the extracellular space to entrap and kill the bacteria (Fuchs *et al.* 2007).

Pathogenic bacteria have developed various strategies to evade this entrapment by neutrophils. NET evasion strategies have been demonstrated for several pathogenic bacteria. For example, the expression of polysaccharide capsules and changing of the cell surface electric charge reduce the entrapment of *Streptococcus pneumoniae* within NETs (Wartha *et al.* 2007). Biofilm formation allows the Gram-negative bacterium *Haemophilus influenzae* to survive within NETs in the middle ear cavity (Hong *et al.* 2009). Furthermore, several pathogens such as Gram-positive *Staphylococcus aureus* (Berends *et al.* 2010), *S. pneumoniae* (Beiter *et al.* 2006), *S. pyogenes* (Sumby *et al.* 2005; Buchanan *et al.* 2006), *S. agalactiae* (Derre-Bobillot *et al.* 2013), *S. suis* (de Buhr *et al.* 2014) as well as Gram-negative pathogens *Vibrio cholera* (Seper *et al.* 2013) and *Aeromonas hydrophila* (Brogden *et al.* 2012) evade entrapment by nuclease-mediated degradation of the NET structure underlining the importance of the expression of one or more nucleases for virulence.

In this study, we focused on the Gram-negative, rod-shaped zoonotic pathogen *Yersinia enterocolitica*, belonging to the family *Enterobacteriaceae*. *Yersinia enterocolitica* causes a range of enteric diseases summarized as Yersiniosis, and is commonly found in the environment. Yersiniosis occurs in different organisms including humans, cattle, deer, pigs and birds (Bottone 1997). The main reservoirs for *Y. enterocolitica* are pigs and pork-derived meat products (Fredriksson-Ahomaa *et al.* 2011; Fredriksson-Ahomaa 2012). The infection occurs after the uptake of contaminated sources such as undercooked meat products (Tauxe *et al.* 1987). The infection with *Y. enterocolitica* causes fever, abdominal pain and diarrhoea (Bottone 1997). The intensity of the symptoms depends on the bacterial strain, the inoculation dosage as well as on the age of the patients, with infected children developing the most severe symptoms. The serotypes O:8, O:9 and O:3 belong to the most important human-virulent serotypes, with O:3 considered to be the most infectious serotype and the most frequent cause of human Yersiniosis (Bottone 1997, 1999; Valentin-Weigand, Heesemann and Dersch 2014).

Various components of *Y. enterocolitica* induce host immune responses; however, the bacterium has established various strategies enabling circumvention of recognition and consequent destruction by immune cells ultimately permitting to establish the infection (Reis and Horn 2010). Interestingly, YadA renders *Y. enterocolitica* sensitive to NET-dependent killing (Casutt-Meyer *et al.* 2010): *Y. enterocolitica* serotype O:9 used in this study was able to induce NETs in a YadA-independent manner. Furthermore, the authors demonstrated via an entrapment assay that only YadA-expressing *Y. enterocolitica* adhere to NETs and are killed. However, it remains unclear whether other serotypes are also able to induce NET formation and/or whether *Y. enterocolitica* are able to escape from NETs.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The following bacteria were used in this study: *Y. enterocolitica* serotype O:8 (8081v; Pepe, Badger and Miller 1994), O:9 (5603; McNally *et al.* 2006) and O:3 (Y1/07; Uliczka *et al.* 2011). Strains were grown on Luria-Bertani (LB) agar plates and in LB medium at 37°C at 200 rpm shaking. For all experiments, bacteria from mid-log phase (OD_{600} 0.5) were washed by centrifugation at 2739 *g* for 10 min at 4°C and resuspended in phosphate-buffered saline (PBS). *Escherichia coli* DH5α carrying pBSU101 ($Spectinomycin^R$, Aymanns *et al.* 2011) was grown overnight in LB with Spectinomycin (100 μg ml^{-1}) and pBSU101 was isolated with the NucleoSpin Plasmid Kit (Macherey-Nagel) according to the manufacturer's instructions.

Neutrophil isolation and NET induction

To investigate the ability of *Y. enterocolitica* serotypes to induce the release of NETs, human neutrophils were isolated from healthy donors by density gradient centrifugation using PolymorphPrep (Axis-Shield, Oslo, Norway). Then, neutrophils were resuspended in RPMI-1640 (PAA, Freiburg, Germany) and plated on coverslips in poly-L-lysine coated 48-well plates (Nunc, Germany) at a concentration of 2×10^5 cells/well. Cells were then infected with bacteria (MOI 1), centrifuged for 5 min at 370 *g* and incubated at 37°C and 5% CO_2 for different time points (0.5, 1, 2, 3 and 4 h). As positive control to stimulate NET formation, 25 nM phorbol 12-myristate 13-acetate (PMA; Sigma, Hamburg, Germany) was used, while untreated neutrophils served as negative control. Finally, cells were fixed with 4% paraformaldehyde (PFA; Roth, Germany).

NET degradation

To analyse, whether *Y. enterocolitica* degrade NETs, 2×10^5 neutrophils/well were pre-stimulated with 25 nM PMA for 4 h as described above. Then, plates were centrifuged at 370 *g* and incubated for 4 h at 37°C and 5% CO_2 following addition of 100 μl bacterial supernatant or washed bacteria (OD_{600} 0.5). Media only (RPMI-1640, PAA, Freiburg, Germany) served as negative control. For the positive degradation control, 0.01 units ml^{-1} of micrococcal nuclease (MN) from *S. aureus* (Worthington, Lakewook, NJ, USA) was added. The plates were incubated for an additional hour at 37°C and 5% CO_2.

To determine the effect of calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions on NET degradation, neutrophils were washed once with HBSS lacking Ca^{2+} and Mg^{2+} ions (PAA, Freiburg, Germany) after the 4 h NET induction. The controls were washed with PBS. To exclude the involvement of protease activity in NET degradation, a control including a protease inhibitor mix (PI; 1.48 μM antipain dihydrochloride, 0.768 μM aprotinin, 10.51 μM leupeptin, 1.46 μM pepstatin A, 50 μg ml^{-1} trypsin-inhibitor,

1 mM phenylmethanesulfonyl fluoride; Sigma, Hamburg, Germany) was added. After the washing procedure, the cells were supplemented with RPMI-1640 (controls), RPMI-1640 with PI or HBSS. The bacterial pellets were also washed with HBSS. Next, the bacterial suspension was centrifuged at 2739 *g* at 4°C for 10 min and resuspended with LB (control), LB with PI or HBSS, respectively. An aliquot of 100 μl of each condition was added to the neutrophils and incubated for 1 h at 37°C and 5% CO_2 followed by fixation with 4% PFA overnight at 4°C or for 10 min at room temperature. Surviving CFU per millilitre was quantified and in Fig. 2, the level of degradation was quantified relative to 1×10^7 CFU.

DNA degradation assay

To assess nuclease activity, MN (New England Biolabs, Frankfurt am Main, Germany) or bacterial supernatants were incubated with DNA substrate. *Yersinia enterocolitica* from mid-logarithmic phase culture (OD_{600} 0.5) or *E. coli* overnight culture (OD_{600} 2.8) were pelleted by centrifugation at 2739 *g* for 10 min at 4°C and the supernatants were harvested. Twenty-two microlitres of the filter-sterilized supernatant was incubated with approximately 300 ng of purified circular plasmid DNA (pBSU101-GFP) in a reaction buffer with Ca^{2+} and Mg^{2+} (0.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 10 mM Tris-HCl; pH 7.4) or without (10 mM Tris-HCl; pH 7.4). After 20 h (*Y. entercolitica*) or 80 min (*E. coli*) at 37°C, 12 μl of the samples with addition of 6 × loading dye (Fermentas, Wohlen, Switzerland) were separated on a 0.8% agarose gel (0.5 μg ml^{-1} ethidium bromide; Sigma, Hamburg, Germany) and visualised with a Benchtop UV Transiluminator (UVP, Axon Lab, Baden, Switzerland) and the TS Image software (UVP).

NET visualisation

NET formation was visualised by fluorescence confocal microscopy as previously described by de Buhr *et al.* (2014). Area statistics were calculated for the complete image from six individual images per sample. Regions without a NET were subtracted from the whole size of the image to determine the resulting proportion of NETs in one image.

Statistical analysis

Data were analysed using Excel 2003 (Microsoft) and GraphPad Prism 5.0 (GraphPad Software). The NET experiments were performed with two replicates in at least three independent experiments. Differences between two groups were analysed by using a paired, one-tailed Student's t-test. The significance is indicated as * $P < 0.05$; ** $P < 0.005$ and *** $P < 0.001$.

RESULTS AND DISCUSSION

Yersinia enterocolitica serotype O:9, O:8 and O:3 induce release of NETs

Three serotypes of *Y. enterocolitica* were incubated with primary blood-derived neutrophils to investigate the ability of *Y. enterocolitica* to induce NET formation. NET formation was visualised by confocal fluorescence microscopy and quantified by counting the NET-releasing nuclei. All serotypes induced NETs (Fig. 1). However, serotype-dependent differences were observed: while a significant induction of NET formation for serotypes O:8 and O:3 was observed already after 0.5 h of incubation (Fig. 1A and B), significant NET induction mediated by serotype O:9 was observed only after 1 h of incubation (Fig. 1C and D). Remarkably, O:3 showed the most NET induction (different compared to negative control at 4 out of 5 time points) compared to the other two strains (different compared to negative control at only 1 or two time points).

We conclude from these data that different serotypes of *Y. enterocolitica* are able to induce NETs. However, the serotype O:3 strain showed a stronger induction of NETs than the others (Fig. 1C). This could be due to different expression patterns of NET-inducing surface proteins. For example, in serotype O:3 strains, but not in the O:9 and O:8 strains the outer membrane protein invasin is expressed in addition to YadA at 37°C (Uliczka *et al.* 2011). Future studies in our lab will focus on the identification of *Y. enterocolitica* NET-inducing factors. Interestingly, presence of NETs distinctly decreased with serotypes O:8 and O:9 after prolonged co-incubation with neutrophils at 2, 3 and 4 h compared to earlier time points (Fig. 1A and C).

These data are in line with a study by Casutt-Meyer *et al.* (2010) reporting that neutrophils treated with *Y. enterocolitica* E40 (Serotype O:9) showed a significant induction of NETs after 60 min, but a reduced amount of NETs after 120 min (Fig. 1A and D). This reduction could be a consequence of degradation as a means to escape from entrapment by NETs. Similarly, *S. suis* showed an early induction of NETs after 30 min and a reduction after 90 min of co-incubation with neutrophils (de Buhr *et al.* 2014). The authors demonstrated that membrane-bound nuclease SsnA, which is partially secreted into the surroundings, is responsible for the NET degradation (de Buhr *et al.* 2014).

Yersinia enterocolitica are able to degrade NETs

To test whether *Y. enterocolitica* are able to degrade NETs, we performed NET degradation assays with washed bacteria as well as bacterial supernatants. Blood-derived neutrophils were stimulated for 4 h with PMA to induce NETs and then incubated with bacterial supernatant or washed bacteria (Fig. 2A). To investigate whether there is a serotype-specific difference, the data with washed bacteria were normalised and degradation was calculated relative to 1×10^7 CFU bacteria (Fig. 2B). MN of *S. aureus* was used as positive control for NET degradation. As shown in Fig. 2B, the result of the NET degradation assay showed a significant reduction of NETs mediated by the washed bacteria in comparison to the control with PMA (Fig. 2C). Degradation was also observed when NETs were incubated with the supernatants of all tested *Y. enterocolitica* serotypes (Fig. 2A and C), indicating that NET-degrading factors were released from the bacteria into the medium. Overall, the strongest degradation was observed for serotypes O:8 and O:9, demonstrating that the ability to resolve NET formation is somewhat reduced in serotype O:3 strains. In general, the supernatants show a more powerful NET-degrading effect compared to the washed bacteria, indicating that secreted factors are predominantly involved in this process.

Gram-negative bacteria, such as *Y. enterocolitica*, have a three-layered cell wall structure: the cytoplasmic membrane, the peptidoglycan layer and the outer membrane through which the secretion systems have to deliver their cargo, such as effector proteins. Pathogenic *Y. enterocolitica* serotypes have a type III secretion (T3SS) as well as a type II secretion (T2SS) system (von Tils *et al.* 2012), through which a variety of secreted effector proteins, such as Yersinia outer proteins (Yops), enter the host cells and affect the organism. These effector proteins may play a role in the degradation of NETs. Another

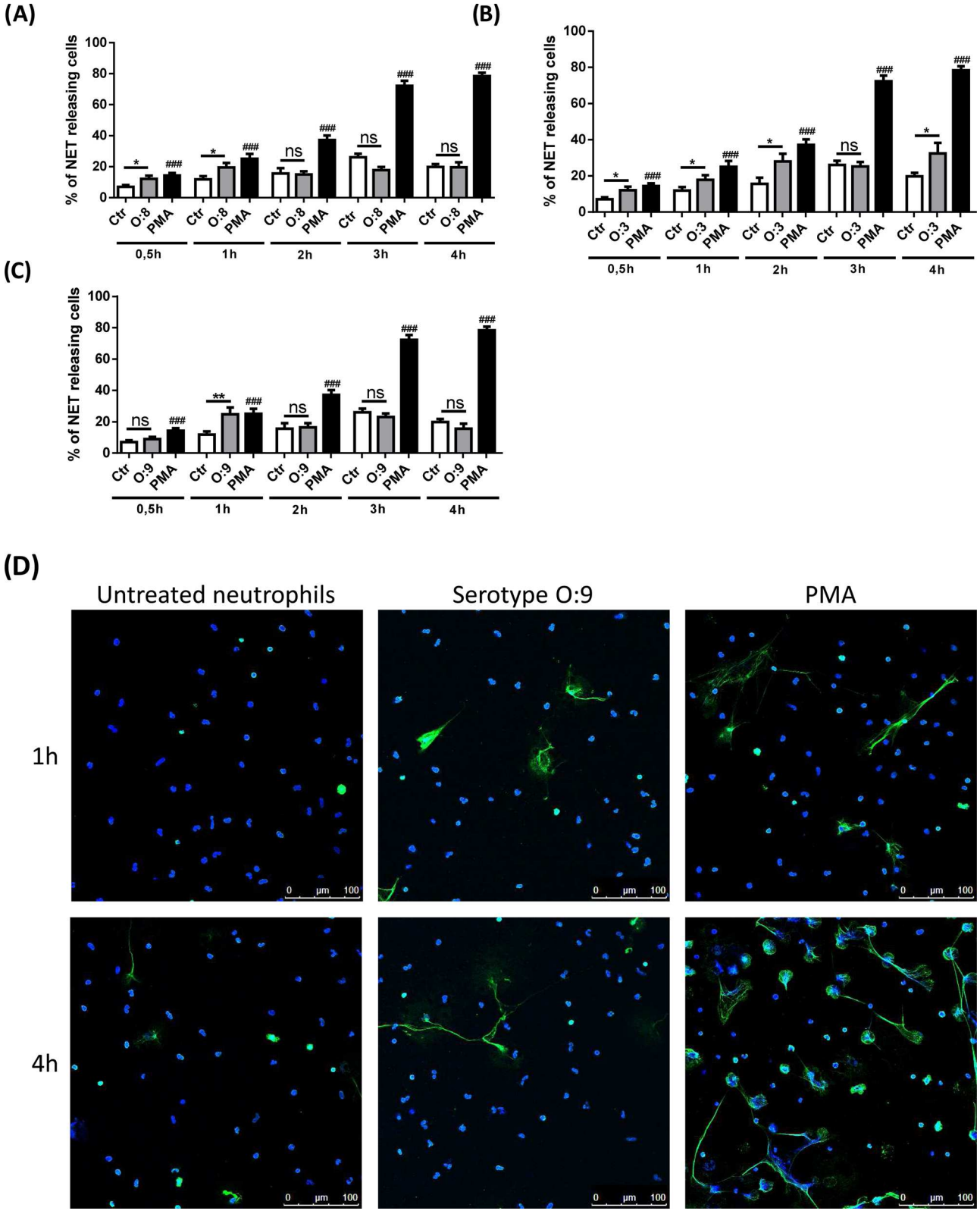

Figure 1. Different *Y. enterocolitica* serotypes induced NET formation over time. Primary blood-derived neutrophils were incubated with washed bacteria (MOI 1) for 0.5, 1, 2, 3 and 4 h. Co-incubation of the cells with O:8 (**A**) and O:3 (**B**) resulted in release of NETs already after 0.5 h serotypes, whereas co-incubation with O:9 (**C**) displayed significant NET release after 1 h. 25 nM PMA was used as a positive control of NET release. Results of four independent experiments, each with six individual images, were analysed using one-tailed Student's *t*-test compared to time-matched untreated control. ns = not significant, $^{*} = P < 0.05$, $^{**} = P < 0.005$, $^{***} = P < 0.001$; PMA treatment was analysed using one-tailed Student's *t*-test compared to untreated control. $^{\#\#\#} = P < 0.001$. (**D**) Representative fluorescent micrographs displaying the NET release of neutrophils co-incubated for 1 h and 4 h with *Y. enterocolitica* serotype O:9. NETs were visualised with a primary H2A-H2B-DNA complex antibody and a secondary Alexa 488-labelled goat-anti-rabbit antibody (green). DNA was stained with DAPI (blue).

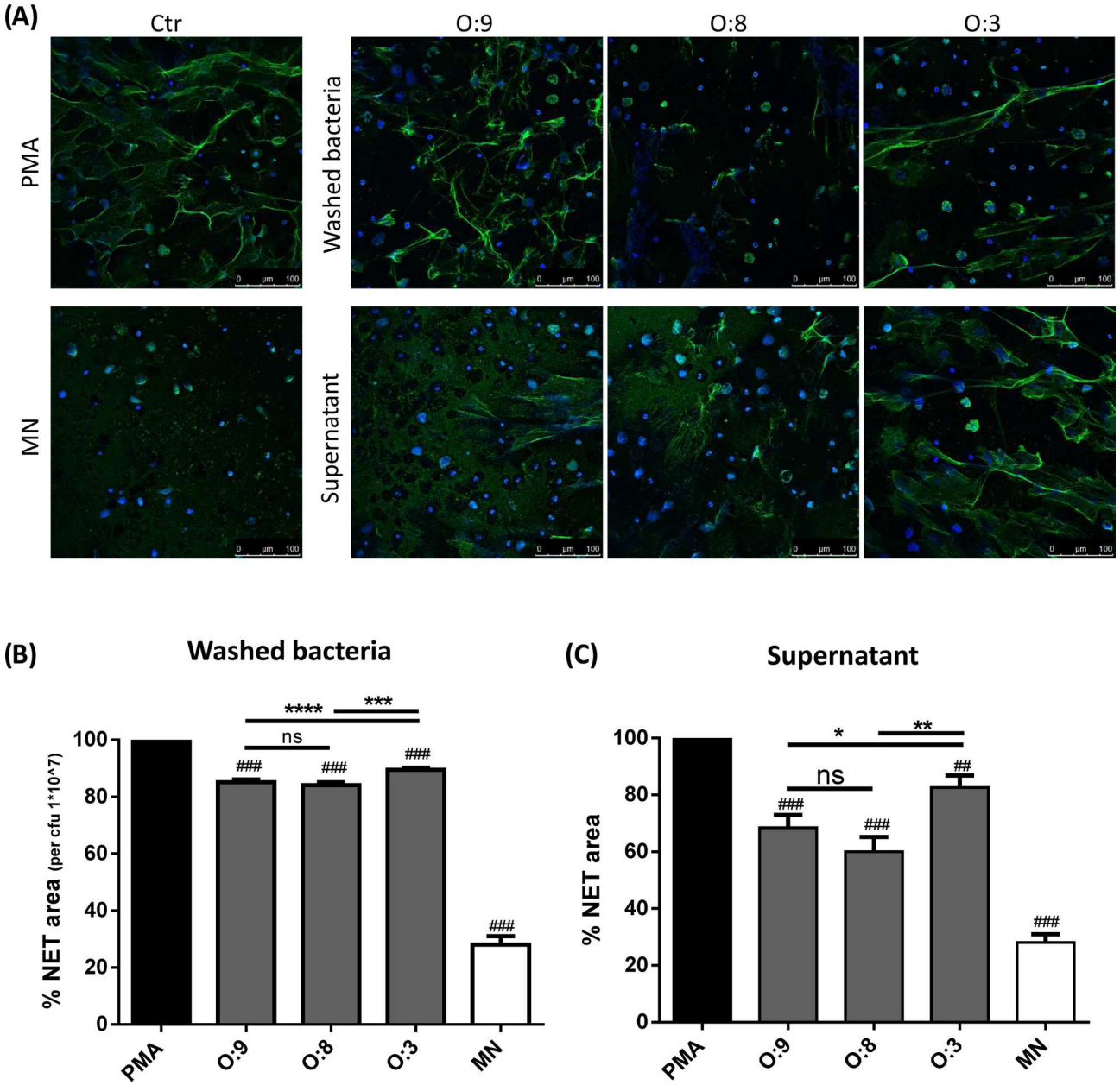

Figure 2. All three *Y. enterocolitica* serotypes exhibited degrading activities resulting in dismantling of NETs. (**A**) Representative fluorescent micrographs of PMA pre-treated neutrophils co-incubated with 0.01 U ml^{-1} MN from *S. aureus*, washed *Y. enterocolitica* strains or *Yersinia* supernatants showed clearly visible degradation of the NET fibres. NETs were visualised with a primary H2A-H2B-DNA complex antibody and a secondary Alexa 488-labelled goat-anti-rabbit antibody (green). DNA was stained with DAPI (blue). Incubation of neutrophil-derived extracellular traps with (**B**) all three washed *Y. enterocolitica* serotypes normalised to a CFU of 1×10^7 and (**C**) supernatants of all *Y. enterocolitica* serotypes displayed degradation of the NETs within 1 h; PMA control was adjusted to 100% NET area. Data of four independent experiments with each six individual images were analysed using one-tailed Student's *t*-test compared to PMA control with $^{###} = P < 0.001$; differences between the serotypes were analysed using one-tailed Student's *t*-test. Ns = not significant, $^{*} = P < 0.05$, $^{**} = P < 0.005$, $^{***} = P < 0.001$.

possible mechanism of pathogens evading NETs could be the production or secretion of nucleases. For *Enterobacteriaceae*, a NET-degrading nuclease has not yet been described. It has recently been discussed that the secretion of a DNA endonuclease could assist *Y. ruckeri* in escaping from NETs through the digestion of the chromatin scaffold (Sallum and Chen 2010). The authors showed an increase in bacterial intracellular and extracellular endonuclease expression after exposure of the bacteria to different concentrations of an antimicrobial peptide called cecropin B. However, a specific NET-degrading effect has not yet been described.

NET degradation by *Y. enterocolitica* is dependent on Ca^{2+} and Mg^{2+} ions

To further investigate whether the detected NET degradation capability of *Y. enterocolitica* was due to a nuclease activity, Ca^{2+} and Mg^{2+} availability was blocked during the 1 h co-incubation time with the neutrophils. Treatment with HBSS lacking Mg^{2+} and Ca^{2+} ions resulted in complete abolishment of the NET degradation in comparison to the control with PMA (Fig. 3A). Furthermore, for all three tested serotypes, there was still significant NET degradation by the washed bacteria after treatment with

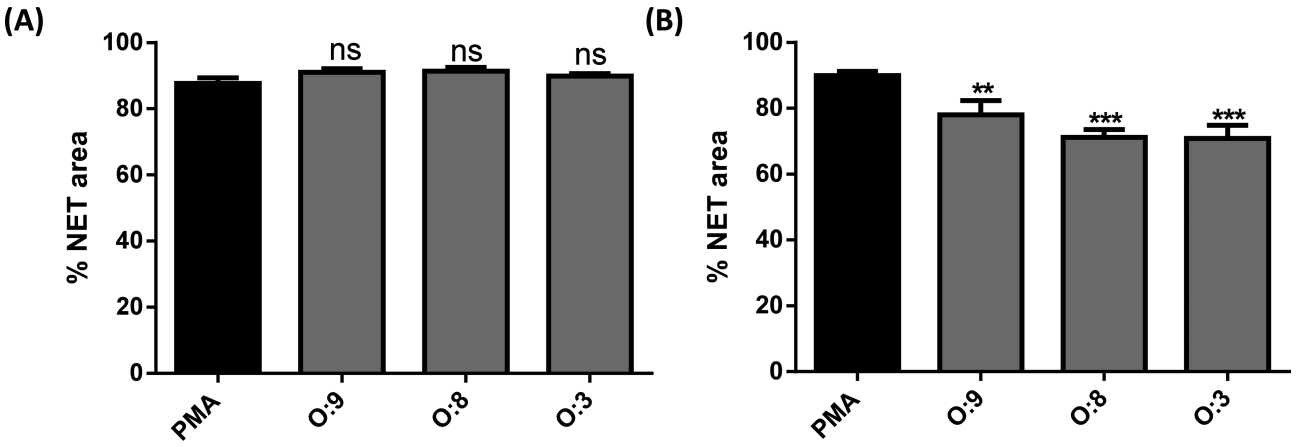

Figure 3. NET degradation mediated by *Y. enterocolitica* serotypes O:9, O:8 and O:3 was dependent on Ca^{2+} and Mg^{2+} ions. (**A**) Depletion of Ca^{2+} and Mg^{2+} ions resulted in a total lack of degradation ability by the *Y. enterocolitica* serotypes within 1 h. (**B**) Treatment of the washed bacteria with protease inhibitor showed no influence on the NET degradation. A comparison between each single serotype and PMA control was performed using one-tailed Student´s *t*-test of three independent experiments with six individual images each. ns = not significant, ** = $P < 0.005$, *** = $P < 0.001$.

protease inhibitor (Fig. 3B). This suggests that proteases are not involved in NET degradation mediated by *Y. enterocolitica*.

Many nucleases require divalent cations such as Ca^{2+} and Mg^{2+} as cofactors. The observed hampered NET degradation capability in the absence of Ca^{2+} and Mg^{2+} (Fig. 3A) led to the hypothesis that a nuclease is involved in NET degradation by *Y. enterocolitica*. However, the availability of these cofactors also influences the expression pattern of virulence factors (Dewoody, Merritt and Marketon 2013), which in turn could indirectly be responsible for NET degradation. Depriving Ca^{2+} from the medium *in vitro* triggers a massive upregulation of T3SS/*yop* gene expression along with secretion of T3SS substrates (Straley and Bowmer 1986; Dewoody, Merritt and Marketon 2013). This results in a varied set of secreted Yops (Dewoody, Merritt and Marketon 2013). In the presence of Ca^{2+}, secretion of early- and middle-phase T3SS substrates into the extracellular milieu is readily observed, whereas the late-phase Yops are only released in large amounts upon Ca^{2+} depletion or contact with host cells (Dewoody, Merritt and Marketon 2013).

Yersinia enterocolitica serotype O:9, O:8 and O:3 showed extracellular nuclease activity

In order to investigate whether *Y. enterocolitica* secretes nucleases, circular plasmid DNA (pBSU101-GFP) was incubated with culture supernatants from the respective *Y. enterocolitica* serotypes. Extracellular nuclease activity was assessed by subsequent analysis of plasmid degradation. Plasmid degradation was observed in all tested *Y. enterocolitica* serotypes in the presence of Ca^{2+} and Mg^{2+}, and serotype O:8 showed the most pronounced extracellular nuclease activity (Fig. 4A). In the absence

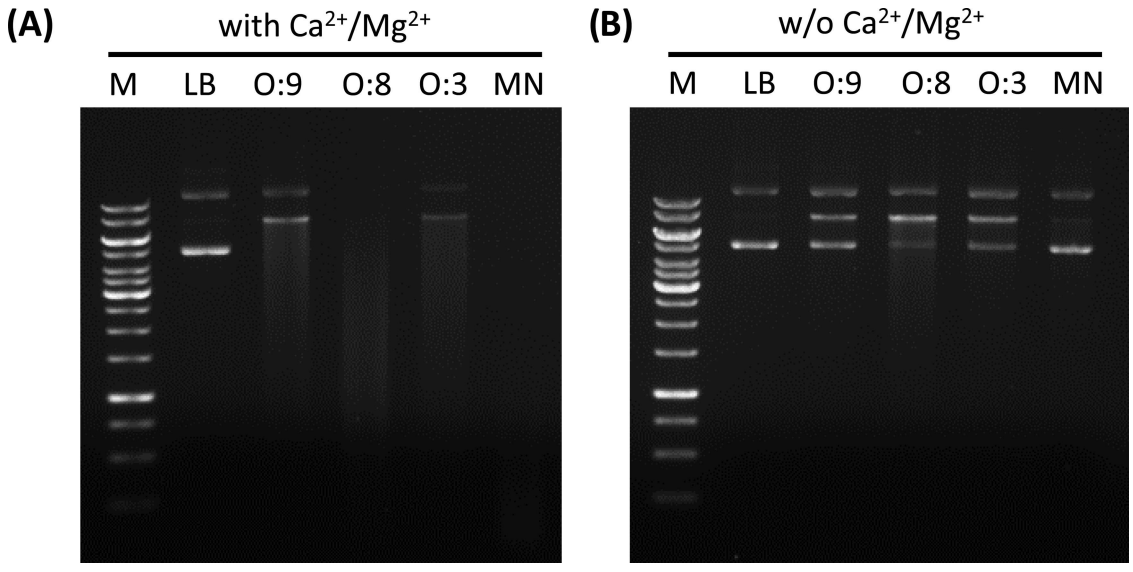

Figure 4. *Yersinia enterocolitica* serotype O:9, O:8 and O:3 exhibit nuclease activities resulting in DNA degradation. Supernatants derived from bacterial cultures grown to mid-exponential phase were assayed for their nuclease activity by addition of circular plasmid DNA. (**A**) Nuclease activity resulted in a shift of supercoiled to linearised plasmid DNA and finally a smear of degraded plasmid DNA in the presence of calcium and magnesium. LB or MN (0.66 gel units ml^{-1}) were used as controls. (**B**) In the absence of calcium and magnesium, almost no DNA degradation could be observed for the tested *Y. enterocolitica* serotypes as well as the MN control. Representative pictures of agarose gels from three independent experiments are shown. M = 1 kb DNA ladder (Fermentas).

Figure 5. Amino acid sequence alignment of the Endonuclease 1 protein (EndA, NucM) sequence of *Y. enterocolitica* with the *V. cholerae* extracellular deoxyribonuclease (Dns). A protein blast of the two sequences yielded the following results: score: 294 bits (752), E-value: 1e-104 and Identity of 67%. (GI:123443619 and GI:550388044, respectively).

of calcium and magnesium, almost no DNA degradation could be observed for the tested *Y. enterocolitica* serotypes as well as the MN control (Fig. 4B). The dependence of DNA degradation on Ca^{2+} and Mg^{2+} is a strong indication that the observed DNA degradation can be attributed to an extracellular nuclease enzyme which is also likely to be responsible for the observed NET degradation (Fig. 2B and C).

In conclusion, our results indicate that three different *Y. enterocolitica* serotypes (O:8; O:9 and O:3) have the ability to induce and degrade NETs. The degradation of NETs occurs by means of a yet uncharacterised mechanism. We highly suspect the activity of a nuclease, similar to what has been described for several other pathogens. As an example for Gram-positive bacteria, nuclease expression was reported for *S. aureus* by using targeted mutagenesis studies (Berends *et al.* 2010) which demonstrated that the nuclease production was associated with delayed bacterial clearance in the lung and an increased mortality after intranasal infection *in vivo*. Beiter *et al.* (2006) showed that *S. pneumoniae* express endonuclease EndA, which can promote the spreading from the upper airways to the lungs and from the lungs into the bloodstream during pneumonia. A recent study showed that Gram-negative *V. cholerae* secretes two nucleases, Dns and Xds, which are responsible for NET degradation and subsequent evasion of the host immune response (Seper *et al.* 2013). A protein blast of the *V. cholerae* Dns against the genome of *Y. enterocolitica* O:8 (8081v) yielded a high match with an expected value of 1e-104 for the *Y. endonuclease* 1 protein (EndA, also known as NucM). The alignment of the two proteins shows regions of conserved amino acids (Fig. 5). This high degree of similarity suggests that *Y. enterocolitica* endonuclease may act in a manner similar to that of *V. cholerae*.

This is the first experimental proof that members of the *Enterobacteriaceae* family are able to degrade NETs, possibly due to a nuclease. To corroborate the results, NET and DNA degradation assays were performed with another pathogenic species of this family, *E. coli* (Fig. S1, Supporting Information). A significant degradation of the NET structure was observed after 1 h incubation of the pre-stimulated neutrophils with supernatant from cultures of a pathogenic *E. coli* strain (Fig. S1, Supporting Information). This strongly suggests that degradation of NETs by secreted nucleases is also a common trait of *Enterobacteriaceae* to avoid neutrophil attacks. Future experiments are directed to identify and characterize the bacterial NET evasion factors and determine their role for the infection process of Yersinia.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

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Conflict of interest. None declared.

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